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Failure of Muscle Myosin Heavy-Chain Gene Expression in Quarter Ascidian Embryos Developed from the Secondary Muscle Lineage Cells

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ABSTRACT—Muscle cells of the ascidian tadpole larva originate from two different lineages, the primary (B4.1 line) and secondary (A4.1 and b4.2 lines) lineages. Experiments with 8-cell embryos have indicated that isolated blastomeres of the primary lineage show autonomous muscle development, whereas blastomeres of the secondary lineage rarely develop the differentiation markers (muscle-specific antigens and specific enzyme activity) in isolation. However, there is the possibility that A4.1 and b4.2 quarter embryos might express a muscle-specific gene but the transcripts might not be translated into proteins, thus we would not be able to detect the muscle differentiation. In order to examine the possibility, four blastomere-pairs (a4.2, b4.2, A4.1, and B4.1 pairs), isolated from the 8-cell embryo of *Halocynthia roretzi,* were allowed to develop into quarter embryos, and the occurrence of transcripts of myosin heavy-chain gene was determined by *in situ* hybridization of whole-mount specimens. The transcripts were evident only in B4.1 quarter embryos and not in A4.1, b4.2 and a4.2 quarter embryos. Thus, the proportion of A4.1 and b4.2 quarter embryos that develop muscle cells does not increase even when examined at the transcriptional level.

INTRODUCTION

A tadpole larva of the ascidian *H. roretzi* contains forty-two unicellular, striated muscle cells (twenty-one cells on each of the right and left sides of the tail). The lineage of the muscle cells is well documented [1, 2]. Twenty-eight muscle cells in the anterior and middle part of the tail originate from a pair made up of the right and left B4.1 cells (posterior-vegetal blastomeres) of the 8-cell embryo, while four cells in the posterior region and ten in the caudal tip region are derived from the A4.1 (anterior-vegetal) and b4.2 (posterior-animal) pairs, respectively. A pair of a4.2 cells (anterior-animal) in the 8-cell embryo does not contribute to formation of muscle. Many investigations have been carried out to elucidate the cellular and molecular mechanisms involved in the specification of muscle cells in ascidian embryos [for recent reviews see 3–8]. The extensive potential for self-differentiation of muscle cells from isolated B4.1 cells has been demonstrated by the assessment of the occurrence of several markers of muscle differentiation [9–14]. Muscle differentiation was also evident in partial embryos developed from isolated a4.2+b4.2+A4.1 cells [12, 15]. By contrast, few of A4.1 and b4.2 quarter embryos developed muscle cells [9, 13–16], suggesting a difference in mechanisms for specification between primary and secondary muscle cells.

Recently, cDNA probes for an ascidian gene for muscle-specific actin [17, 18] and a similar gene for myosin heavy chain [19, 20] have been prepared. Analysis by Northern blotting and *in situ* hybridization with the aid of these probes has shown that transcripts of the two genes are detectable at stages as early as the gastrula stage [17–20]. It is highly probable that the genes for muscle-specific actin and myosin heavy chain become transcriptionally

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activated at around the time of the initiation of gastrulation. Therefore, it remains a possibility that the differentiation of muscle cells in A4.1 and b4.2 quarter embryos might be evident at the transcriptional level but, for unknown reasons, the transcripts might not be translated into polypeptides. Thus, we would not be able to detect the development of muscle by assessing the occurrence of muscle-specific antigens and the histochemically detectable activity of acetylcholinesterase. The present study was, therefore, designed to determine whether the quarter ascidian embryos that originate from isolated A4.1 and b4.2 pairs differentiate as muscle cells at the transcriptional level. Using the technique of *in situ* hybridization, we examined the occurrence of transcripts of the gene for myosin heavy chain in quarter embryos of *H. roretzi*. The transcripts were detected only in B4.1 quarter embryos and not in A4.1, b4.2 and a4.2 quarter embryos.

MATERIALS AND METHODS

Embryos

Naturally spawned eggs of *Halocynthia roretzi* were fertilized with a suspension of sperm from another individual. Fertilized eggs were raised in filtered seawater at about 13°C. At this temperature, they developed to gastrulae about 9 hr after insemination and to early-tailbud embryos after about 15 hr of development; they hatched about 35 hr after fertilization.

Isolation of blastomeres and production of quarter embryos

Eggs were dechorionated with sharpened tungsten needles about 20 min after fertilization. Naked eggs were cultured to the 8-cell stage in 1.0% agar-coated petri dishes. Only 8-cell embryos with a normal appearance were used for the isolation of blastomeres. The four blastomere-pairs of the 8-cell embryo (a4.2, b4.2, A4.1, and B4.1 pairs) were separated with a glass needle under a dissecting microscope. They were reared to quarter partial embryos. Features, such as the location of polar bodies, the configurations of the blastomeres, and the distribution of pigments were used to assess orientation of the embryos. Isolated blastomere-pairs were cultured separately in 24-well multiwells plates (Falcon) coated with 1% agar. Millipore-filtered (pore size, 0.2 μm) seawater containing 50 μg/ml streptomycin sulfate was used for culture of dechorionated eggs and isolated blastomere-pairs. Quarter embryos were cultured until normal embryos hatched and then they were fixed for *in situ* hybridization.

In situ hybridization of whole-mount specimens

In situ hybridization with a digoxigenin-dUTP-labeled DNA probe was carried out on whole-mount specimens basically according to the method described by Makabe *et al.* [20]. The original cDNA probe used for *in situ* hybridization was a 1.6-kb EcoRI fragment of cDNA that encodes a part of myosin heavy chain of *H. roretzi* [19]. Labeling of the 1.6-kb fragment with digoxigenin-dUTP (Boehringer Mannheim, Germany) was carried out by the random primer method according to the protocol from Boehringer.

Quarter embryos, as well as middle-tailbud embryos (as controls), were fixed for 30 min in ice-cold ethanol : acetic acid (3 : 1, v/v). The fixed specimens were washed extensively with PBT (phosphate-buffered saline that contained 0.1% Tween 20) and treated with 10 μg/ml proteinase K in PBT for 30 min at 37°C. After washing with PBT, the specimens were post-fixed with 4% paraformaldehyde in PBT for 20 min at room temperature, and then they were washed again with PBT. Specimens were then treated with PBT : hybridization buffer (1 : 1, v/v) for 10 min at room temperature and then with hybridization buffer alone for 10 min at room temperature. After a 1-hr prehybridization at 42°C, the specimens were hybridized with the digoxigenin-labeled DNA probe for 18 hr at 42°C. The hybridization buffer consisted of 5×SSC (1×SSC comprises 0.15 M NaCl and 0.015 M Na_3 citrate), 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin, 50% formamide, and 0.1% Tween 20. After hybridization, the solution was gradually exchanged for PBT. The samples were then incubated for 1 hr with 500 μl of Dig-AP conjugate (polyclonal antibodies raised in sheep against digoxigenin-Fab fragments, conjugated to alkaline phosphatase; Boehringer Mann-

heim) diluted in PBT (1:2000). After washing with PBT and then with colour-developing buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the samples were transferred into 1 ml buffer contained 4.5 μl NBT (nitroblue tetrazolium salt) and 3.5 μl X-phosphat (5-bromo-4-chloro-3-indol phosphate) solutions. Colour was allowed to develop for about 1 hr, and the reaction was stopped by addition of stop solution (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Samples were treated with a mixture of benzyl alcohol : benzyl-benzoate (1:2, v/v) [21] so that the embryos became transparent and the reaction products could be easily distinguished.

RESULTS AND DISCUSSION

The four blastomere-pairs of the 8-cell embryo of *H. roretzi* were separated and allowed to develop into quarter embryos. They were fixed at the time when control, intact embryos hatched, and the occurrence of transcripts of the gene for myosin heavy chain in the quarter embryos was examined by whole-mount hybridization *in situ*. In each of hybridization experiments, the quarter embryos were processed with control middle-tailbud embryos. After all hybridizations, the control middle-tailbud embryos showed distinct positive signals in the differentiating muscle cells on the right and left sides of the tail (Fig. 1A). Isolated a4.2 and b4.2 cells gave rise to partial embryos that looked like permanent blastulae covered with transparent tunic (Fig. 1B, C) while A4.1 and B4.1 quarter embryos consisted of a tail-like cluster of smaller cells to which larger cells were attached (Fig. 1D, E).

Results of the experiment can be summarized by reference to Table 1 and Figure 1. In the first series of experiments, all of the twenty-five B4.1 quarter embryos examined showed distinct expression of the gene for myosin heavy chains (Fig. 1E, F). The positive staining was detected only in the larger cells (Fig. 1E, F). The total number of positive cells was around 25, but the exact number could not be determined. By contrast, none of the A4.1 quarter embryos (0/22; Fig. 1D) and none of the b4.2 quarter embryos (0/27; Fig. 1C) showed positive signals for the occurrence of the trans-

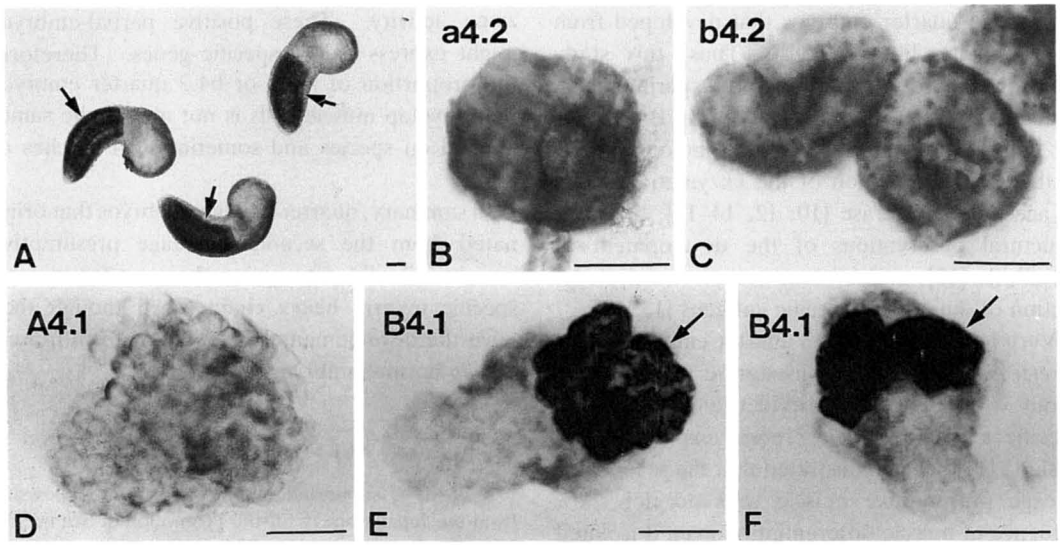

FIG. 1. Detection of transcripts of the gene for muscle-specific myosin heavy chain by *in situ* hybridization using a digoxigenin-labeled DNA probe and whole-mount preparations. (A) Control early-tailbud embryos showing distinct expression of the gene for the muscle-specific protein in differentiating muscle cells on the right and left sides of the tail (arrows). (B) a4.2 quarter embryo, (C) b4.2 quarter embryos, (D) A4.1 quarter embryo, and (E, F) B4.1 quarter embryos. The presence of transcripts of the gene for the muscle-specific protein is evident only in the B4.1 quarter embryos (arrows). Scale bars represent 50 μm in all photographs.

TABLE 1. Occurrence of myosin heavy chain mRNAs in quarter embryos of the ascidian *Halocynthia roretzi*

Experiment	Number of embryos expressing the transcripts			
	a4.2	b4.2	A4.1	B4.1
I	0/18	0/27	0/22	25/25
II	0/32	0/38	0/47	43/43
total	0/50	0/65	0/69	68/68

cripts of the gene for the muscle-specific protein (Table 1). The transcript was undetectable in the a4.2 quarter embryos (0/18; Fig. 1B, Table 1).

A year later, we carried out the second series of experiments, and the results were the same as those of the first one. The transcripts for myosin heavy-chain gene were detected only in all of the forty-three B4.1 quarter embryos (Table 1). The gene expression was not observed in A4.1 (0/47), b4.2 (0/38) and a4.2 (0/32) quarter embryos (Table 1).

The present results demonstrated that, even when muscle differentiation is assessed by use of a specific cDNA probe, the gene transcripts can be detected only in quarter embryos that originated from the primary-lineage presumptive muscle cells and not in quarter embryos that developed from the secondary-lineage cells. Thus, this study confirms the results of previous experiments in which differentiation of muscle cells in B4.1 quarter embryos was assessed by morphology [9], by histochemical detection of the enzymatic activity of acetylcholinesterase [10, 12, 14–16], by ultrastructural observations of the development of myofibrils [11], and by immunocytochemical detection of the muscle-specific antigens [13, 14].

Very few A4.1 and b4.2 quarter embryos of *H. roretzi* develop acetylcholinesterase activity (only about 3%) [16] or showed evidence of the muscle-specific antigen [13]. A recent experiment by Nishida [14] has demonstrated that the secondary-lineage presumptive muscle cells do not show evidence of muscle differentiation even if isolated from 64-cell embryos. The present study has confirmed such a tendency of non-autonomous development of the secondary-lineage presumptive muscle cells at the transcriptional level. Cellular mechanisms for the determination of the fate of muscle cells in the ascidian embryo, may differ between the primary and secondary lineages. As recently discussed by Davidson [8], the differentiation of the primary-lineage presumptive muscle cells takes place *autonomously* and is controlled by intrinsic factors, while the specification of progenitors of the secondary-lineage b4.2 and A4.1 cells occurs *conditionally* (subject to regulation by extrinsic factors). However, in some experiments about 15% of b4.2 quarter embryos of *H. roretzi* developed the muscle-specific antigen (myosin heavy chain protein) [13]. In addition, in the case of *Ascidia ceratodes,* Meedel *et al.* [15] reported that all of the A4.1 quarter embryos develop acetylcholinesterase, although less than 5% of the animal-half embryos (a4.2+b4.2) showed the enzyme activity. These positive partial-embryos might express muscle-specific genes. Therefore, the proportion of A4.1 or b4.2 quarter embryos that develop muscle cells is not always the same, depend on species and sometimes on batches of eggs.

In summary, quarter ascidian embryos that originated from the secondary-lineage presumptive muscle cells did not express the gene for muscle-specific myosin heavy chain, even though they have the developmental potential to form muscle during normal embryogenesis.

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